Pak. J. Bot., 35(2): 243-248, 2003.

PLASMID BORNE ANTIBIOTIC RESISTANCE FACTORS AMONG INDIGENOUS *KLEBSIELLA*

SHEIKH AJAZ RASOOL, AFSHEEN AHMAD, SADIA KHAN AND ABDUL WAHAB

Department of Microbiology,
University of Karachi, Karachi, Pakistan.

Abstract

About fifty indigenous clinical *Klebsiella* were isolated and identified on the basis of morphology, growth, and biochemical characteristics. Fifty two percent were identified as *K. oxytoca*, 42% as *K. pneumoniae* and 6% as *K. ozaenae*. All the isolates offered different resistance patterns (determined by medium incorporation-replica method) against antibiotics including ampicillin, streptomycin, gentamicin, ofloxacin, tetracycline and chloramphenicol. Some of the representative isolates lost the antibiotic resistance after acridine orange mediated curing. Two methods (uninterrupted tube method and solid substrate mediated mating) were tried for *in vivo* gene transfer studies for determining the conjugative/ transferable nature of the drug resistance plasmid markers. In only one out of five mating (filter paper) experiments, chloramphenicol and ampicillin resistance markers were transferred to the recipient *E. coli* MD40 cells. The rest of the plasmid borne markers were non-conjugative/nontransferable. Conjugative plasmids carry a tremendous potential to disseminate resistance markers to distant recipient cells.

Introduction

Klebsiella pneumoniae (and some related species) is an opportunistic Gram-negative rod pathogen involved in the outbreaks of nosocomial infections (in intensive care units), lower respiratory, urinary tract and burn wound infections. Infact, nosocomial infections associated with *Klebsiella* spp., have shown an increase in most part of the world (Neu, 1992). The members of this genus have also been linked to epidemics of diarrhoea, because some strains appear to have acquired plasmids from *E. coli* (that code for the heat labile and heat stable enterotoxins (Ewing, 1986).

The wide spread use of antimicrobial agents has failed to eradicate microbial infections despite their benefits. Antibiotic resistant bacteria have been a source of ever-increasing therapeutic problem. Continued mismanaged selective pressure has contributed towards the emergence of multiple drug resistant bacteria and that has been regarded as an inevitable genetic response to antimicrobial therapy (Cohen, 1992). The antibiotic resistant mutants that arise spontaneously are generally resistant to only one antibiotic. However, *Klebsiella* spp., exhibit simultaneous resistance to multiple drugs (Gutmann *et al*., 1985). The R plasmids offer resistance to antibiotics and are transmissible from one cell to another by direct cell contact. Conjugation (direct *in vivo* gene transfer) is a convenient method of transferring drug resistant genetic determinants among intra and inter generic bacterial populations. A surveillance study has demonstrated the emergence of highly resistant *Klebsiella* spp., in urinary and respiratory tract infections (Bonafede & Louis, 1997).

The present study was undertaken to address and assess the MDR problems with respect to the indigenous clinical *Klebsiella*.

Materials and Methods

Bacterial isolates and identification: Fifty clinical isolates from sputum, nasal mucosa, and throat swab were collected from various hospitals and laboratories of Karachi. They were identified using MacConkey and TSI medium of Bio M (as per Holt *et al*., 1994).

Determination of antibiotic resistance patterns: The antibiotics (Sigma) used (in 10mg/mL stocks) in this study include ampicillin (amp), chloramphenicol (cm), gentamicin (gm), ofloxacin (ofx), streptomycin (sm) and tetracycline (tc). Antibiotic resistance patterns were studied by replica technique (Lederberg & Lederberg, 1952).

Curing experiments: Representative strains were subjected to acridine orange (Merck) mediated plasmid (bearing resistance markers) elimination (Hahn & Chiak, 1976; Marcelo *et al*., 1993).

***In vivo* gene transfers:** Representative MDR *Klebsiella* spp., were used as donors for the possible transfer of resistance markers to a standard recipient *E. coli* MD40 strain (Clewel, 1993; Selvarathnan & Gealt, 1993).

Results

About 50 clinical isolates of *Klebsiella* collected from different hospitals and clinical laboratories of Karachi were screened for drug resistance pattern. Resistance level of the isolates against different antibiotics at different concentrations is shown in Table 1 and Figure 1. Table 2 indicates the cumulative antibiotic resistance pattern offered by the isolates at 500μg/mL. Acridine orange was used as curing agent during this study for the elimination of plasmid. Effects of plasmid curing on the drug resistance determinants of *Klebsiella* isolates are depicted in Table 3. Plasmid conjugation experiments were performed by filter paper mating. Accordingly, *Klebsiella pneumoniae* AS 22 was used as the donor strain while *E. coli* MD 40 as the recipient for this experiment. Table 4 shows the drug resistance pattern of donor, recipient and the transconjugant.

Discussion

The present communication relates to the isolation-identification (based on morpho-cultural and biochemical considerations) of the indigenous clinical *Klebsiella.* Majority (52%) of the isolates were identified as *K. oxytoca* followed by *K. pneumoniae* (42%) and *K. ozaenae* (6%). All the isolates were found to be associated with different clinical manifestations ranging from enteritis in children, upper respiratory tract infections (and pneumonia), meningitis and urinary tract infections (in children and the adults). Infact, *Klebsiella pneumoniae* has been well implicated in severe (often fatal) pneumonia (Ewing, 1986).

Table 1. Antibiotic resistance offered by *Klebsiella* isolates at different concentrations.

	Percentage of resistant strains at different concentrations (μg/mL)						
Antibiotics	**10**	**50**	**100**	**200**	**300**	**400**	**500**
Ampicillin	70	68	64	62	56	23	18
Chloramphenicol	82	76	70	62	56	25	17
Gentamicin	00	00	00	00	00	00	00
Ofloxacin	02	02	02	02	02	02	02
Streptomycin	54	36	36	30	28	28	24
Tetracycline	04	04	04	04	00	00	00

Table 2. Antibiotic resistance patterns (at 500μg/mL) of *Klebsiella* isolates.

S. No.	Resistance pattern	No. of isolates	Percentage (%)
1.	A	18	36
2.	AC	17	34
3.	ACG	1	2
4.	ACGO	1	2
5.	ACGOS	12	24
6.	ACGOST	0	0

Table 3. Effect of acridine orange mediated plasmid curing on the antibiotic resistance pattern of *Klebsiella* isolates.

Isolate no.	Resistance pattern	
	Pre-curing	**Post-curing**
AS-02	SCAT	-
AS-18	SACGOT	SA
AS-22	ACOT	-
AS-33	SACOT	SA

Table 4. Antibiotic resistance behaviour of *Klebsiella pneumoniae* AS-22 (donor), *Escherichia coli* MD40 (recipient) and the transconjugant.

Culture	Strain	Resistance markers	Miscellaneous marker (s)
Donor	*K. pneumoniae* AS-22	ACOT	Lac^-
Recipient	*E. coli* MD-40	S	Lac^+
Transconjugant	*E. coli* TC-22	A*C*S	Lac^+

Key:
A= Ampicillin, C= Chloramphenicol, G= Gentamicin, O= Ofloxacin, T= Tetracycline, S= Streptomycin,
Lac^+ = Positive regulation of lac operon (lactose fermentor).
Lac^- = Non activity of *lac* operon (lactose non-fermentor).
* * = Transferred markers.

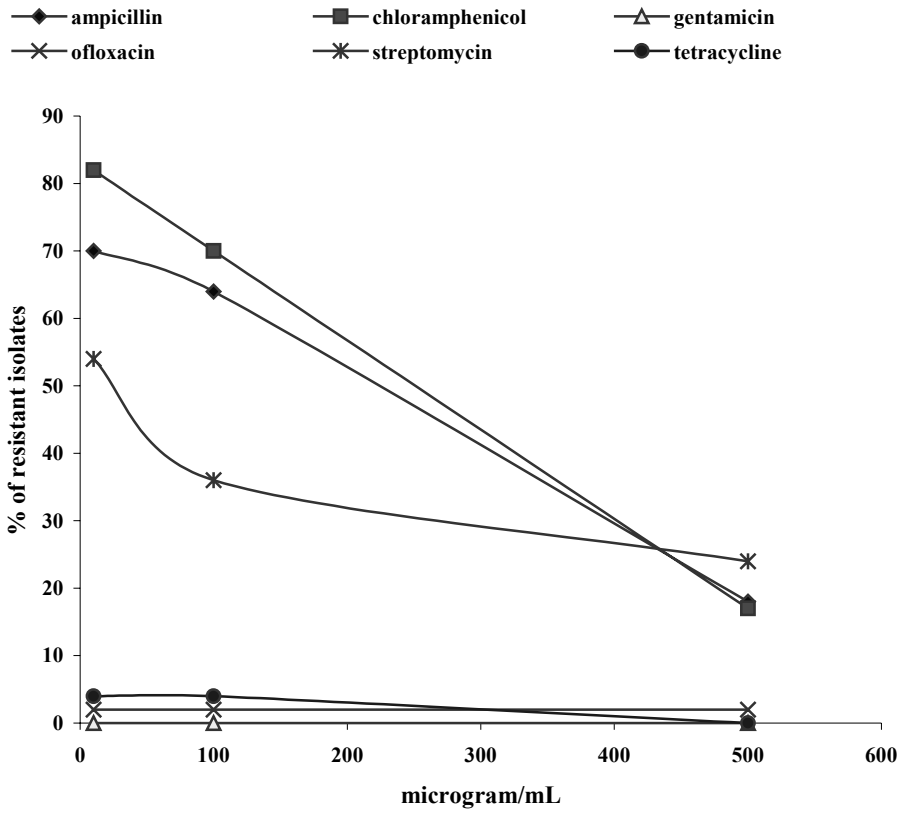

Fig. 1. Occurrence of antibiotic resistance in *Klebsiella* isolates.

All the isolates were screened for their resistance profiles to a battery of six antibiotics representing different groups, depending upon their mode of action (Table 1). Surprisingly, the resistance (at one or the other concentration) has been shown against all the antibiotics used in this study. Table 2 indicates the statistical presentation of resistance patterns (at 500μg/mL of different antibiotics) by different *Klebsiella* isolates. Accordingly, gentamicin was found most effective followed by ofloxacin; while chloramphenicol, ampicillin, and streptomycin were found least effective. Five different antibiotic resistance patterns were identified. Increasing drug resistance trend has been reported earlier (Rasool *et al*., 1993; Ghazala, 2000). In *Klebsiella pneumoniae*, an unusual class of multiple drug resistance (MDR) mutants exhibiting simultaneous resistance to the structurally unrelated antibiotics were isolated by selection of resistance to β-lactam antibiotics or to fluoroquinolones (eg. ofloxacin) by Sanders *et al*., (1984). Such MDR mutants were found to have reduced levels of at least one major outer membrane protein (Omp) and to exhibit reduced uptake of chloramphenicol (Gutmann *et al.,* 1985). Analysis of the bacterial collections from the pre-antibiotic era indicates that although plasmids were present in some of the strains but did not harbour antibiotic resistance genes (Chakrabarty *et al*., 1990).

The location (chromosomal or extra chromosomal) of drug resistance determinants was confirmed by plasmid curing strategies. In this connection, acridine orange mediated curing was performed (Table 3). Resultantly, some of the resistance markers were stably lost (excluding streptomycin and ampicillin in terms of the MDR klebsiella strains; thereby showing the chromosomal location of these two markers). Plasmids with traits of both resistance and conjugation are known as RTF (Davies, 1994). Plasmid conjugation is an important mechanism of disseminating drug resistance among bacterial populations. During the present studies, solid substrate (millipore filter paper) mating experiments were undertaken (using a representative *K. pneumoniae* as donor and *E.coli* MD40 as the recipient strain). The transconjugants had shown the stable transfer of the resistance markers of ampicillin and chloramphenicol. Plasmid DNA mediated transfer of resistance determinants has earlier been reported as well (Joan, 1997). Such broad host-range transferable plasmids play an important role in the spread of antibiotic resistance. However, conjugation (physical mating) strategies do not allow the pushable plasmids which need the sex pili provided by an independent F-plasmid (Rasool, 1992). In such circumstances, transformation may work well; infact, the race to develop agents to overcome the resistance mechanism is one that man may never win, but the resistance trends should be kept under check through intensive research leading to novel and alternative drugs therapies.

Acknowledgement

Research grant received from the University of Karachi is gratefully acknowledged.

References

Bonafede, M. and B.R. Louis. 1997. Emerging antibiotic resistance. *J. Lab. Clin. Med*., 130(6): 558-566.

Chakrabarty, A.M., S.G. Dastidar and M. Ganguli. 1990. DNA as contaminant in antibiotics and its capacity to transfer bacteria to drug resistance. *Ind. J. Exp. Biology*, 28: 58-62.

Cohen, M.L. and R.V. Auxe. 1992. Drug resistant *Salmonella* in the United States: an epidemiologic perspective. *Science*, 234: 964-970.

Clewel, D.B. 1993. *Bacterial conjugation*. Plenum press, New York.

Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science*, 264: 375-381.

Ewing, W.H. 1986. *Identification of Enterobacteriaceae* (4^{th} edition). Elsevier, Netherlands.

Ghazala, S.S. 2000. *Plasmids of indigenous pseudomonads: characterization and gene manipulation*. M. Phil thesis, University of Karachi. pp-1-190.

Gutmann, L., R. Williamson, R. Moreau, M.D. Kitzis, E. Collatz, J.F. Acar and F.W. Goldstain. 1985. Cross resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alterations in the outer membrane proteins of *Klebsiella*, *Enterobacter* and *Serratia*. *J. Infect. Dis*., 151: 501-507.

Hahn, F.E. and J. Chiak. 1976. Elimination of resistance determinants from, R factor by intercalating compounds. *Antimicrobial Agents Chemother.*, 9: 77-80.

Holt, G.T., R.N. Krieg, P.H.A. Sneath, T.J. Staley and T.S. Williams. 1994. *Bergey's Manual of Determinative Bacteriology*. 9th edition. (Williams and Wilkins, Baltimore, Md., USA). pp176-211.

Joan, S. 1997. Worry grows as antibiotic resistant bacteria continue to gain ground. JAMA, 278: 2049-2050.

Lederberg, J. and F.M. Lederberg. 1952. Replica plating and induced selection of bacterial mutants. *J. Bacteriol*., 63: 399-401.

Marcelo, E.T., A.A. Luis and H.C. Jorge. 1993. Virulence plasmids. In: *Plasmids: A practical approach* (Ed.): K.G. Hardy. Oxford University press, Oxford. pp. 95-118.

Neu, L.H. 1992. The crisis in antibiotic resistance. *Science*, 257: 1054-1073.

Rasool, S.A. 1992. Bacteriocins: the protein antibiotics. (Fazlee Sons, Karachi) p.15.

Rasool, SA., B. Ahmed and A. Iqbal. 1993. Multiple drug resistance among indigenous clinical staphylococci: *In vivo* manipulation and characterization of the R-plasmids. *Proc. Pak. Congr. Zool*., 13: 439-447.

Sanders, C.C., W.F. Sandro, R.V. Goering and V. Werner. 1984. Selection of multiple antibiotic resistance by quinolones, β-lactam and aminoglycosides with special reference to cross-resistance between unrelated drug classes. *Antimicrob. Agents Chemother.*, 26: 797-801.

Selverathnan, S. and M.A. Gealt. 1993. Transcription of ColE1 Ap mbcC induced by conjugative plasmids from twelve different incompatibility groups. *J. Bacteriol*., 175: 6982-6987.

(Received for publication 20 September 2002)